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STUDIES ON THE ACTIVATION OF LYSINE-2,3-AMINOMUTASE BY (—)-S-ADENOSYL-L-METHIONINE

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SUMMARY

To investigate the physiological significance of the activation of lysine-2,3-aminomutase by S-adenosylmethionine in *Clostridium* SB_4, the content of the sulfonium compound has been measured in this microorganism with a new isotope dilution technique. 0.57 μmole of *S*-adenosylmethionine per g of wet bacterial cell have been detected, indicating saturating cellular concentrations of the sulfonium compound with respect to lysine mutase (the apparent K_m of the enzyme with respect to *S*-adenosylmethionine is $2.8 \cdot 10^{-8}$ M).

To study the mechanism of such activation, several *S*-adenosylmethionine analogues have been tested: among them only *S*-adenosyl-(5')-3-methylthiopropylamine (decarboxylated *S*-adenosylmethionine) exerts an appreciable effect on the enzyme activity, while *S*-adenosyl-L-(2-hydroxy-4-methylthio)butyric acid, *S*-inosyl-L-methionine, *S*-inosyl-L-(2-hydroxy-4-methylthio)butyric acid, *S*-methyl-L-methionine and its deaminated derivative did not modify the reaction rate to any extent. The data suggest that both amino groups of the molecule are involved in the activation. In addition, experiments with (—)-*S*- and (±)-*S*-adenosylmethionine demonstrate that the steric configuration of the sulfonium pole is not related to the activation mechanism.

Equilibrium dialysis experiments between purified enzyme and *S*-adenosyl-[*carboxy*-^{14}C]methionine or *S*-adenosyl[*Me*-^{14}C]methionine failed to show any measurable binding between the sulfonium compound and the protein.

Precipitation of the enzyme with trichloroacetic acid in the presence of *S*-adenosyl[*Me*-^{14}C]methionine confirmed the apparent absence of a tight binding between the labeled carbon of the sulfonium compound and the enzyme.

INTRODUCTION

Lysine-2,3-aminomutase, a bacterial enzyme responsible for the reversible conversion of lysine to β-lysine[1], has been recently purified by Chirpich *et al.*[2] from *Clostridium* SB_4. The activity is pyridoxal-phosphate dependent and is specifically and

markedly stimulated by *S*-adenosyl-L-methionine: in absence of the sulfonium compound only 30% of the maximal activity can be reached. 4 moles of *S*-adenosylmethionine per mole of enzyme are required for maximal activity[2].

In addition, the sulfonium compound and its analogue thioether (*S*-adenosyl-L-homocysteine) protect the protein from sodium dodecylsulfate dissociation, as it has been demonstrated by Zappia *et al.*[3].

The role of *S*-adenosylmethionine as methyl donor in transmethylations is widely recognized[4,5], while its function as cofactor has been demonstrated only in few instances, namely in the biosynthesis of methionine[6,7] and in the pyruvate: formate-lyase reaction[8].

As far as the mechanism of lysine mutase activation, an allosteric effect is not likely since there is no evidence of sigmoid kinetics[2]. On the other hand the existence of several enzymes methylating the lysine and arginine residues of proteins[9–11] suggests an activation mechanism involving the methylation of the protein, which could in turn cause a favourable conformational change. An adenylylation mechanism, in analogy with glutamine synthetase from *Escherichia coli*[12–13], could also be postulated, even if the role of *S*-adenosylmethionine as adenylylating agent has not been so far demonstrated[14].

To investigate the nature of such activation, equilibrium dialysis experiments between labeled *S*-adenosylmethionine and the purified enzyme have been performed. In addition several analogues and derivatives of *S*-adenosylmethionine have been tested as activating agents.

Finally, to investigate if such activation is operative *in vivo*, the cellular content of *S*-adenosylmethionine in *Clostridium* SB_4 has been measured with a new procedure developed in this laboratory[15].

MATERIALS AND METHODS

Lysine-2,3-aminomutase was purified from *Clostridium* SB_4 according to the method of Chirpich *et al.*[2]. The preparation was homogeneous on the basis of disc gel electrophoresis performed according to the method of Davis[16]. The enzyme assay was performed according to the method of Chirpich and Barker[17]. *S*-Adenosyl[*Me*-^{14}C]-methionine was prepared by biosynthesis with yeast from L-[*Me*-^{14}C]methionine (Amersham)[18–19], *S*-adenosyl[*carboxy*-^{14}C]methionine was synthesized from L-[*carboxy*-^{14}C]methionine (Amersham) and isolated by ion-exchange chromatography[19]. The compounds were chemically and radiochemically pure as judged by thin-layer chromatography and paper ionophoresis followed by radioactivity scanning[20]. (±)-*S*-Inosyl-L-methionine was prepared by methylation of *S*-inosyl-L-homocysteine with methyl iodide[20], *S*-adenosyl-L-(2-hydroxy-4-methylthio)butyric acid and *S*-inosyl-L-(2-hydroxy-4-methylthio)butyric acid were obtained by deamination of *S*-adenosylmethionine with nitrous acid[20]. (±)-*S*-Adenosylmethionine was synthesized by chemical methylation of *S*-adenosyl-L-homocysteine with methyl iodide[21]. *S*-Methyl-L-methionine sulfonium salt was obtained as iodide by the method of Toennies and Kolb[22].

The purity of *S*-adenosylmethionine extracted from *Clostridium* SB_4 was checked by ultraviolet spectrophotometry, by thin-layer chromatography on silica gel plates and by high-voltage ionophoresis[20].

Radioactivity was measured in a Packard Liquid Scintillation Counter Model 3380 equipped with an absolute activity analyzer. A standard solution of 0.4% 2,5-diphenyloxazole, 0.04% 2,2'-*p*-phenylbis-(4-methyl-5-phenyloxazole) in a mixture of equal volumes of toluene and ethanol was used. Quenching was corrected with external standardization. Radioactivity on paper strips or silica gel plates was detected by a radioactivity scanner Actigraph III, Model 1002 (Nuclear Chicago).

Absorbances were determined on a Zeiss spectrophotometer Model PMQ II. Proteins were estimated by the method of Lowry *et al.*[23] with bovine serum albumin as standard.

RESULTS

Estimation of S-adenosylmethionine in Clostridium SB_4

5 g of *Clostridium* SB_4 cell paste (harvested in the logarithmic growth phase) were homogenized at 4 °C with 19 ml of 1.5 M perchloric acid and 25 μl of *S*-adenosyl-

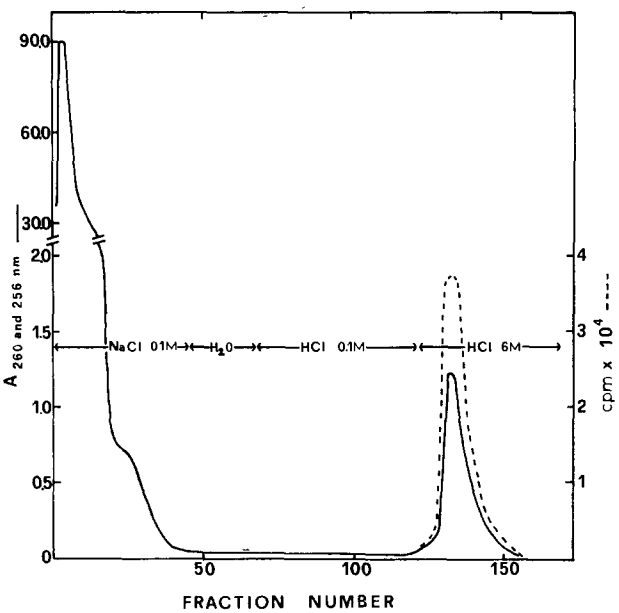

Fig. 1. Elution pattern of ion-exchange chromatography of *S*-adenosylmethionine extracted from *Clostridium* SB_4.

[*Me*-^{14}C]methionine (0.8 mM, $4.2 \cdot 10^7$ cpm/ml). The mixture was allowed to stand for 1 h to extract the sulfonium compound. After centrifugation (9000 × *g*, 15 min) the supernatant was neutralized at 2 °C with a saturated solution of $KHCO_3$; solid potassium perchlorate was removed by filtration and the supernatant was added to a Dowex 50 column (Na^+ form, resin bed 1 cm × 13 cm). The eluting agents were used in the following order: 0.1 M NaCl, water, 0.1 M HCl and 6 M HCl, with a flow rate of 30 ml/h; water and 0.1 M HCl were used to remove the excess of NaCl, 6 M HCl to elute *S*-adenosylmethione: the elution pattern is reported in Fig. 1. The fractions (4

ml each) were scanned for ultraviolet absorption at 260 nm (NaCl and water) or at 256 nm (0.1 M and 6 M HCl), and for radioactivity. Fractions 130–146, containing a peak of ultraviolet-absorbing material associated with radioactivity, were pooled, desiccated under reduced pressure and redissolved in 250 μl of water.

A qualitative analysis of the compound(s) present was performed with thin-layer chromatography and high-voltage ionophoresis. With both methods only one ultraviolet, ninhydrin- and chloroplatinate-positive compound was detected, in a single spot coincident with the peak of radioactivity and with a reference sample of *S*-adenosylmethionine (Fig. 2). The ultraviolet absorption spectrum of the compound is

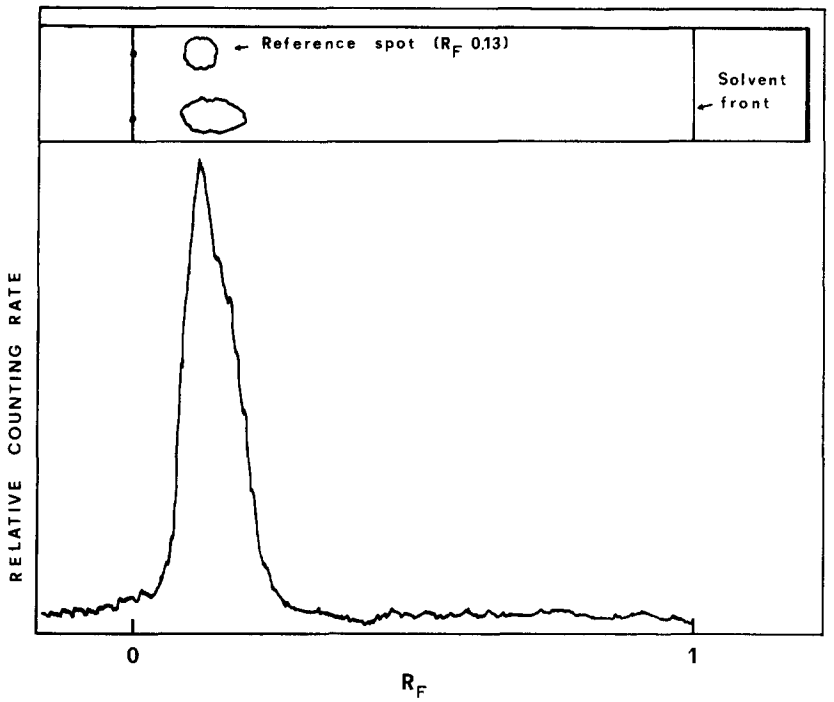

Fig. 2. Radioactivity scanning of thin-layer chromatogram of *S*-adenosyl[*Me*-^{14}C]methionine diluted with *S*-adenosylmethionine from *Clostridium* SB$_4$.

reported in Fig. 3: it is coincident with a spectrum of reference *S*-adenosylmethionine. The specific radioactivity of the ultraviolet-absorbing fractions compared with the specific radioactivity of "carrier" *S*-adenosylmethionine added to the perchloric acid extract permitted one to calculate the amount of the compound present in the bacterial cell[15]: an average of 0.57 μmole/g wet tissue has been obtained from three separate determinations.

Activation by S-adenosylmethionine analogues

Various sulfonium derivatives of *S*-adenosylmethionine were tested for their ability to activate lysine mutase. The results are reported in Table I. Synthetic (±)-*S*-

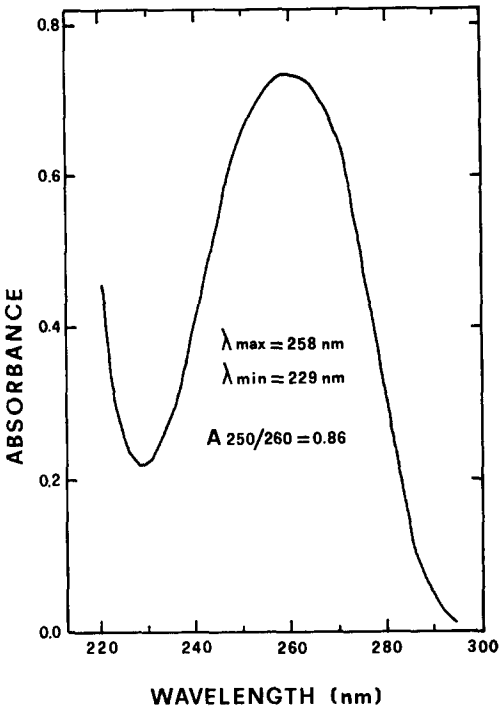

Fig. 3. Ultraviolet absorption spectrum of *S*-adenosylmethionine from *Clostridium* SB_4.

TABLE I

ACTIVATION OF LYSINE MUTASE BY *S*-ADENOSYLMETHIONINE ANALOGUES AND DERIVATIVES

The enzyme (1.65 units/mg) was activated and assayed according to the method of Chirpich and Barker[17]. The concentration of sulfonium compounds added to the assay solution was 4 μM. Activity is expressed as the number of units in the assay.

Additions	*Activity (units × 10⁴)*	*Relative activity*
None	23	100
(−)-*S*-Adenosyl-L-methionine	60	261
(±)-*S*-Adenosyl-L-methionine	61	265
(±)-*S*-Inosyl-L-methionine	24	104
(−)-*S*-Inosyl-L-(2-hydroxy-4-methylthio)butyric acid	23	100
(−)-*S*-Adenosyl-(5′)-3-methylthiopropylamine	58	252
(−)-*S*-Adenosyl-L-(2-hydroxy-4-methylthio)butyric acid	24	104
S-Methyl-L-methionine	24	104
4-Dimethylsulfonium-L-(2-hydroxy)butyric acid	23	100

adenosylmethionine exerted the same effect as the natural (—)-isomer. In addition only *S*-adenosyl-(5')-3-methylthiopropylamine showed significant activity.

The replacement of the 6-amino group of adenine moiety and/or replacement of 2-amino group of the methionine moiety by an hydroxyl group resulted in complete loss of activity. Also the substitution of adenosine moiety by a methyl group (*S*-methyl-L-methionine and its deaminated derivative) resulted in quantitative loss of activity.

Binding experiments

To identify the sulfonium molecule groups participating in the binding to the protein, experiments of equilibrium dialysis between *S*-adenosyl[*Me*-^{14}C]methionine and the enzyme have been devised. The results reported in Table II demonstrate that after 2 h dialysis the equilibrium has been reached. The experiments have been performed either with the sulfonium compound present in the two half cells at zero time,

TABLE II

EQUILIBRIUM DIALYSIS BETWEEN *S*-ADENOSYL[*Me*-^{14}C]METHIONINE OR *S*-ADENOSYL[*carboxy*-^{14}C]-METHIONINE AND "ACTIVATED" LYSINE MUTASE

The experiments have been performed in multicavity dialysis cells (5 units, 60 μl volume each cell. To the half cells A were added: 20 μl of activated enzyme (8 μM, 2.3 mg/ml)) in standard Tris–HCl buffer containing 0.4 μmole of KCl; to the half cells B were added: 20 μl of *S*-adenosyl-[*Me*-^{14}C]methionine (specific radioacivity $4 \cdot 10^7$ cpm/μmole) or 20 μl of *S*-adenosyl[*carboxy*-^{14}C]-methionine (specific radioactivity $2.3 \cdot 10^6$ cpm/μmole) at the indicated concentrations in standard Tris–HCl buffer containing 0.4 μmole of KCl. The dialysis was performed at 37 °C in a rotatory shaker to prevent concentration gradient formation at membrane interface. At the indicated times aliquots of 10 μl were removed with a 10-μl Hamilton syringe and radioactivity was assayed as indicated under Materials and Methods.

	S-Adenosylmethionine concentration in half cell B at time 0 (μM)	*Radioactivity in half cell A (cpm)*			*S-Adenosylmethionine concentration in half cell A after 12 h (μM)*
		Dialysis time:			
		10 min	*2 h*	*12 h*	
S-Adenosyl[*Me*-^{14}C]-methionine	4	730	1 600	1 610	2
	16	2 800	6 430	6 425	8
	40	7 200	16 160	16 140	20
	80	14 200	32 000	32 120	40
S-Adenosyl[*carboxy*-^{14}C]-methionine	0.8	8 300	18 200	18 300	0.4

or by adding it only in one half cell, the enzyme being in the other (Table II). In both cases and with various relative concentrations of *S*-adenosyl[*Me*-^{14}C]methionine (Table II) no difference in radioactivity has been found between the two half cells at equilibrium. Also with *S*-adenosyl[*carboxy*-^{14}C]methionine no measurable difference has been detected.

Additional experiments in which "activated" lysine mutase was precipitated with trichloroacetic acid in the presence of *S*-adenosyl[*Me*-^{14}C]methionine failed to show any measurable binding between the sulfonium compound and the enzyme: lysine mutase (2.3 mg/ml; 1.65 units/mg) was activated for 1 h at 30 °C according to the procedure of Chirpich and Barker[17]; 100 μl of enzyme were then transferred to a 1-ml tube containing 50 μl of 15 μM *S*-adenosyl[*Me*-^{14}C]methionine ($7 \cdot 10^5$ cpm/ml) in

"standard" Tris–HCl buffer, pH 7.8[2]. After 20 min incubation at 30 °C, 0.2 ml of 2% trichloroacetic acid was added and the precipitated protein was collected over a millipore filter. The precipitate was washed with 1% trichloroacetic acid to remove excess of labeled compound and the filter was dried and counted for radioactivity. No measurable radioactivity was detected in two separate experiments.

DISCUSSION

S-Adenosylmethionine concentration in *Clostridium* SB_4 (0.57 μmole/g cells) is unusually high if compared to other microorganisms[18,24], with the exception of *Candida utilis* and *Saccharomyces cerevisiae*[24]. Much lower concentrations have also been found in animal tissues[15]. Since an apparent K_m for *S*-adenosylmethionine of $2.8 \cdot 10^{-8}$ M has been calculated for lysine mutase[2], it appears that the cellular amounts of the cofactor are highly saturating with respect to the enzyme. Therefore limited variations of cellular concentrations of sulfonium compound should not affect the reaction rate *in vivo* and an activation function more than a regulatory one can be attributed to the cofactor.

As far as the activation mechanism, a methylation of the enzyme by *S*-adenosylmethionine could be postulated also considering the role of the sulfonium compound as the most important biological methyl donor[5]. Furthermore, several protein methylases have been characterized[25,26] and in some cases a relationship between the methylation state of the protein and its function has been suggested[27,28]. The results of the binding experiments with *S*-adenosyl[*Me*-^{14}C]methionine seem to exclude the possibility of enzyme methylation.

Lack of binding of *S*-adenosyl[*carboxy*-^{14}C]methionine could suggest a splitting of the sulfonium compound and binding of the only adenosine moiety to the enzyme. The possibility that *S*-adenosylmethionine could act as adenylylating agent has been postulated by Cantoni[14], but not yet been proved in the literature.

The experiments with the sulfonium analogues show that activation is unrelated to the configuration of the sulfonium pole of *S*-adenosylmethionine: in fact both (−)-*S*- and (±)-*S*-adenosylmethionine are equally effective (see Table I). Since it has been demonstrated that in methyl transfer reactions the only (−)-isomer is the active one[21], this finding also indirectly tends to exclude a methylation of lysine mutase. Finally lack of activity of 2-deamino, 6′-deamino and 2,6′-deamino derivatives, infers interactions of the two amino groups of *S*-adenosylmethionine with lysine mutase. The carboxyl group of methionine seems not to be involved as a binding site since the decarboxylated derivative exhibits a significant activity.

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